Fungal Pretreatment of Corn Fiber for Butanol Fermentation

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Abstract

Depleting worldwide fossil fuels and the political advantages of increasing energy independence motivate research in renewable energy. One important characteristic of a new fuel is the ability to directly replace gasoline within the current infrastructure. Fermentation of sugars into alcohols such as butanol will meet this need. A promising strain of bacteria, clostridium acetobutylicum, converts hexose and pentose sugars into acetone, butanol, and ethanol. Butanol has a high energy density and can directly replace gasoline in combustion engines. For biofuels such as butanol to become a realistic alternative, an abundant source for sugars needs to be used. One potential source is lignocellulose or biomass. Lignocellulose consists of three subcategories of molecules; cellulose, hemicellulose, and lignin. Cellulose is a polymer with glucose subunits and hemicellulose has several types of sugar subunits, mainly pentose. Lignin is made up of different organic compounds which, when broken down, inhibit the growth of clostridium. The main intent of this research is to use different types of fungi to decompose the lignin layer and depolymerize the cellulose. The two types of fungi used were *Trichoderma reesei* (cellulase producer) and *Phanerochaete chrysosporium* (lignin decomposer). The two types of fungi were grown on corn fiber, individually and in a co-culture, with the intention to decrease the difficulties of the proceeding hydrolysis and fermentation. The experiment analyzed the ability of the two aforementioned fungi to break down the lignocellulose and lead to fermentable media. The carbohydrates in biomass are an abundant source of stored chemical energy. By finding a way to improve lignocellulosic depolymerization, the technology is available for the downstream processes to make the process economical.

Acknowledgments

This project would not have been possible without those whom have helped me out greatly. I would like to thank Dr. Shang-Tian Yang for giving me direction and allowing me to work in his lab. He has been incredibly welcoming by allowing me to attend research group meetings and has always encouraging me to further my knowledge. I would like to thank Jie Dong, my mentor in the lab. Jie has shown me many lab techniques, protocol, and how to use the analytical equipment. He has answered countless questions and has always guided me in the right direction.

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Introduction

Energy

Energy is at the center of anthropogenic activity. Without the availability of stored energy, transportation and commerce would not be the same. A vast majority of the stored energy used for transportation is from fossil fuels. The energy expended to extract these fuels is increasing, resulting in a decreased energy return as seen in Figure 1.

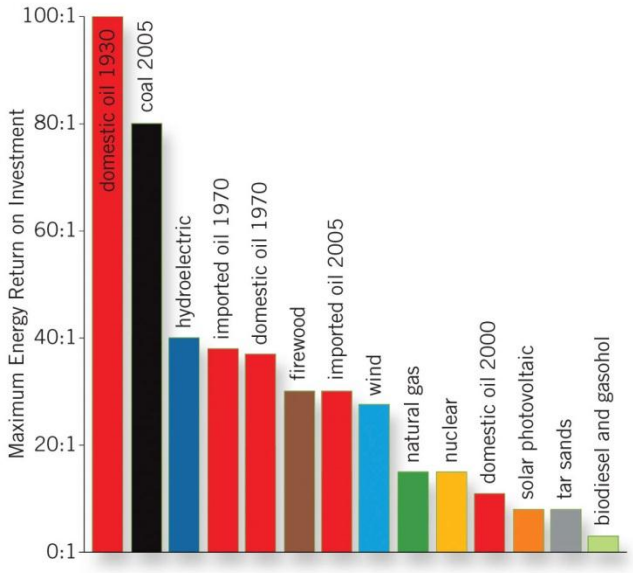

Figure 1: Energy return on investment of several sources [4]

The trend of fossil fuels becoming more energy intensive to extract is likely to continue due to the oil reserves becoming less localized. The energy return on renewable energy is likely to increase with new technologies. As the ease of fossil fuel extraction diminishes, new energy sources will become increasingly competitive.

The earth receives most of its energy from solar insolation which providing about $2.7*10^{24}$ J per year [15]. Of this energy, a portion is stored in chemical bonds via photosynthesis. Photosynthesis is a process in which plants convert the energy from photons into a hydrogen ion gradient across a cell

membrane. This gradient is converted into chemical energy via the production of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) from adenosine diphosphate (ADP) and nicotinamide adenine dinucleotide phosphate (NADP+), respectively. These high energy molecules then are used to produce carbohydrates from carbon dioxide and water. A portion of these high energy carbohydrates are used in cellular respiration and the rest are stored [15]. Some of the excess carbohydrates are then synthesized into biopolymers and stored as lignocellulose; lignin, cellulose, and hemicellulose. Advantages of using lignocellulose instead of starch as the substrate for fermentation are the abundance and it is an agriculture waste instead of a food [16]. Biomass, defined as the total weight of an organism, in plants is made up of a large percent of lignocellulose therefore these polysaccharides represent a great opportunity.

Fossil fuels are derived from plant biomass that has been slowly changed over millions of years resulting in a high energy dense fuel [15]. The energy stored in the fossil fuels comes from the energy that is present in the chemical bonds of lignocellulose. Two main reasons for using the newly stored chemical energy rather than fossil fuels exist; it is renewable and more environmentally friendly. Nature dictates where fossil fuels are located. With the reserves becoming harder and harder to reach the energy return is diminishing. Once the process takes the same amount of energy to extract and convert into fuel as is received from the combustion, the process will no longer be viable. While it may be some time until renewable energy is more efficient, the issue of sustainability is currently driving renewable energy research.

If a car is combusting a carbon based fuel then one of the products will be carbon dioxide. The reason that fossil fuel is widely accepted as causing global climate change is the extra carbon dioxide placed into the atmosphere. A good way to think of the carbon cycle is in terms of how long ago a specific carbon atom was in the atmosphere. With fossil fuels this time is millions of years. The biomass carbon was sequestered from the atmosphere in the plants lifetime. For crops like switch grass and corn

fiber this is likely about a year and for trees it is longer. Since the cycle time for biomass is relatively short the concentration of atmospheric carbon does not appear to have a net change.

The path of energy can be followed from the sun to plants store of carbohydrate polymers. This is where fossil fuel and biomass separate. Fossil fuels have been reacted for millions of years under pressure and in the presence of microorganisms to form high energy dense molecules. The biomass may be fermented to produce biofuels. These biofuels are renewable and have less impact on global climate change. Both fossil fuel and biomass have the same beginnings, but the time scale makes fossil fuel result in a great flux of atmospheric carbon. This research will discuss fermentation; one method of harnessing the energy present in biomass.

Fermentation

Mankind has been brewing beer and fermenting wine for centuries. In this process yeast, a microorganism, metabolizes sugars creating ethanol and carbon dioxide. A similar process is utilized in making fuel grade ethanol. Yeasts are able to achieve a high titer[1], but some issues are present. First yeast only oxidizes hexose sugars, wasting all of the pentose sugars from the hemicellulose [14]. Another problem yeast has is a physical property of the product. Ethanol is corrosive and is not as energy dense as gasoline. An alternative molecule is butanol, a 4-carbon alcohol. Butanol is less corrosive, more energy dense, and a direct replacement for gasoline [11]. A species of bacteria, *Clostridium acetobutylicum*, can ferment hexose and pentose sugars into Acetone, Butanol, and Ethanol (ABE). The metabolic pathway for *C. acetobutylicum* can be seen in Figure 2.

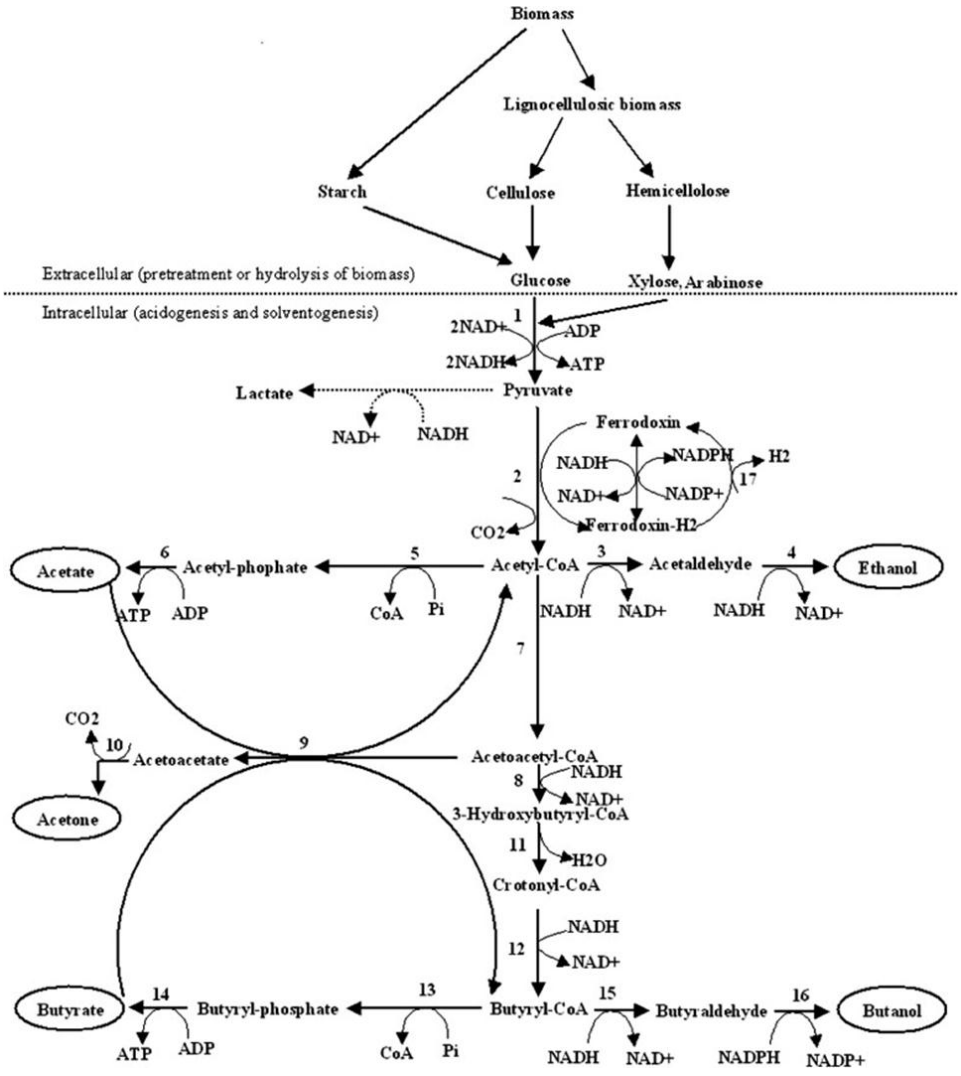

Figure 2: Metabolic pathway for *C. acetobutylicum*[10]

C. acetobutylicum can convert glucose and xylose into pyruvate. From pyruvate, five products exist; Acetic acid, butyric acid, acetone, ethanol, and butanol. Initially, during acetogenesis, the flux is to acetic acid and butyric acid. Then the flux shifts and the acids are converted into the solvents. This phase is called solventogenesis.

Butanol has potential to be utilized as a biofuel. One aspect holding it back is the availability of an inexpensive and abundant substrate. With the ability to metabolize pentose sugars, *C. acetobutylicum* has a distinct advantage over yeast in the potential to utilize a greater number of lignocellulosic sugars.

Pretreatment

Lignocellulose, seen in Figure 3, consists of three main components; lignin, cellulose, and hemicellulose.

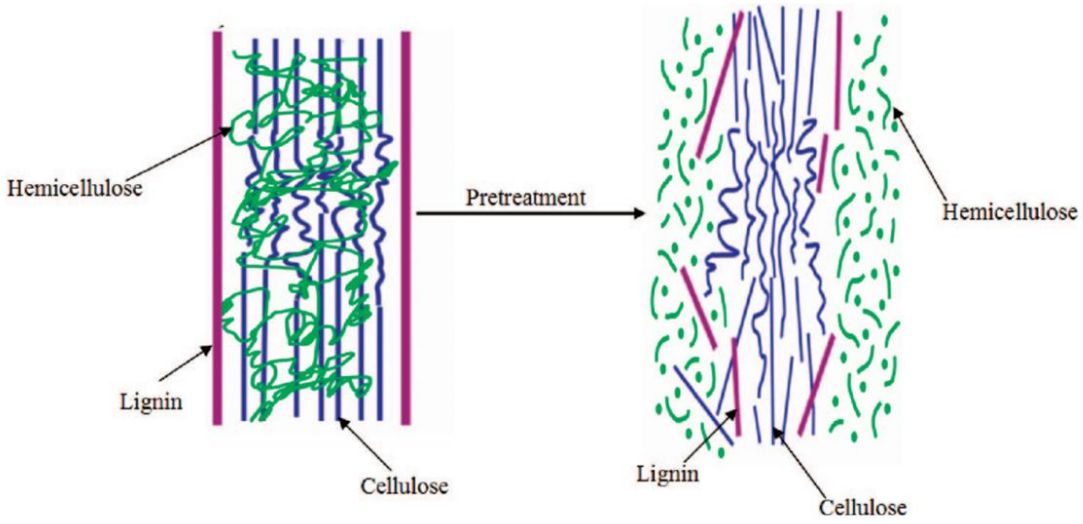

Figure 3: Lignocellulose composition [10]

The lignin layer is a protective barrier and the cellulose and hemicellulose are on the inside. The main purpose of pretreatment is to make the sugars in the cellulose and hemicellulose accessible for hydrolysis. This may be accomplished in many different ways including steam explosion, Ammonia Fiber Explosion (AFEX), CO2 explosion, ozonolysis, acid hydrolysis, organosolv, pyrolysis, pulsed, electrical field, biological treatments [10]. Biological pretreatment can be used to degrade the lignin layer and also breakdown the cellulose. Two types of fungi that can accomplish these tasks are *Trichoderma reesei* and *Phanerochaete chrysosporium,* respectively.

T. reesei is a filamentous fungus that is able to produce several types of cellulase and hemicellulase enzymes. Several different gene locations are known to produce cellobiohydrolase, endo-1,4-glucanase, ß-glucosidase, xylanase and several other biomass degrading enzymes [3].

Cellobiohydrolase starts at the end of a cellulose chain and cleaves the bonds producing disaccharides. ß-glucosidase hydrolyzes the dimers into monomers.

P. chrysosporium, a white rot fungus, is a lignin degrading organism. Studies have been conducted on the ability of *P. chrysosporium* to degrade lignin from different biomass sources including wheat straw and poplar [7] [8]. The effects of several culture parameters, such as aeration, oxygen concentration, and molarity of nitrogen, have been studied [9].

Acid is also able to partially hydrolyze lignocellulose and allow enzymes access to the necessary sites. Acid will result in extra cost and may produce inhibitors. Inhibitors are molecules which will prevent the *C. acetobutylicum* from properly producing ABE. Some of the inhibitors can be seen in

Figure 4.

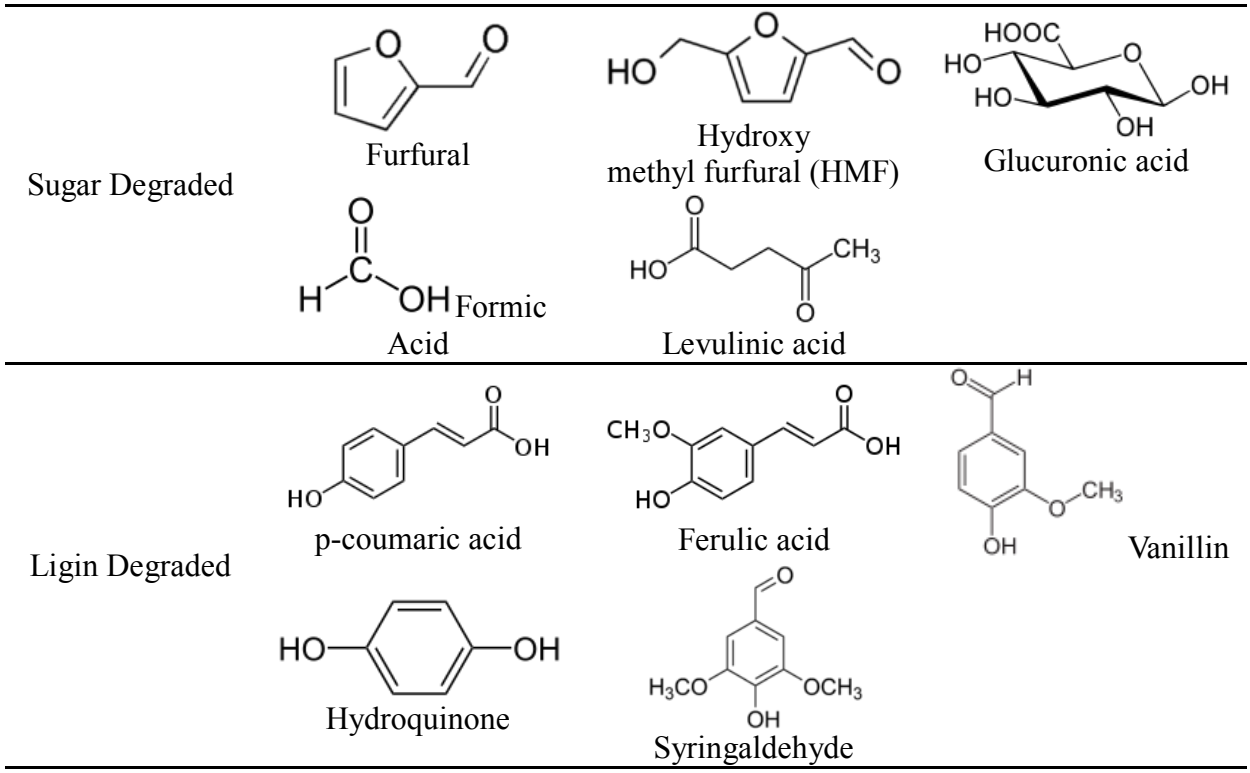

Figure 4: Fermentation inhibitors and their sources[2]

The figure shows that some of the inhibitors are derived from lignin and others are derived from sugars.

The trend of energy consumption and increasing difficulties in extracting fossil fuel are signs that renewable energy is likely to be needed in the future. For a fuel to be easily integrated into the current infrastructure, it must be liquid and have a high energy density. One way to make such a fuel is fermentation. With fermentation, the sugars that will be metabolized can come from lignocellulose. One problem with lignocellulose is the difficulty in hydrolyzing the sugar into fermentable medium. Biological pretreatment is promising in the ability of fungi such as *P. chrysosporium* to degrade the lignin and thus reduce subsequent lignin degraded inhibitors. Another promising aspect is the ability to use *T. reesei* to secrete enzymes in-situ and reduce a major cost, cellulase enzymes. With the added cost cutting of biological pretreatment, the process may become closer to competing with fossil fuel.

Materials and Methods

Fungi Seed cultures-

Phanerochaete Chrysosporium (NRRL 6370) and Trichoderma Reesei (NRRL 3653) were obtained from the United States Department of Agriculture. Potato agar plates were made by mixing potato agar (40 g/L) in distilled water, heating mixture in an autoclave (121 °C, 15 psig) for 30 min, then pouring into petri dishes. The fungi were smeared on the plates and cultured for 7 days. After 7 days, both fungi covered their agar plate.

Medium formulation

The following solutions were made with distilled water, except the experimental medium which was made with hydrolysate. The minerals were made into a concentrated stock solution and added at the start of the fermentation.

Fungal treatment medium – Dextrose (10 g/L), $CaCO_3$ (0.4 g/L), KH_2PO_4 (2 g/L), $(NH_4)_2SO_4$ (1.9 g/L), $MgSO_4 \cdot 7H_2O$ 0.1 g/L, $FeSO_4 \cdot 7H_2O$ 0.015 g/L, $CaCl_2 \cdot 2H_2O$ 0.015 g/L, $MnSO_4 \cdot H_2O$ 0.01 g/L, $CoCl_2 \cdot 6H_2O$ 0.02 g/L, $ZnSO_4 \cdot 7H_2O$ 0.002 g/L

Clostridium Growth Medium (CGM) – Dextrose (50 g/L), $(NH_4)_2SO_4$ (2 g/L), K_2HPO_4 (0.50 g/L), KH_2PO_4 (0.50 g/L), yeast extract (1 g/L), $MgSO_4 \cdot 7H_2O$ 0.1 g/L, $FeSO_4 \cdot 7H_2O$ 0.015 g/L, $CaCl_2 \cdot 2H_2O$ 0.015 g/L, $MnSO_4 \cdot H_2O$ 0.01 g/L, $CoCl_2 \cdot 6H_2O$ 0.02 g/L, $ZnSO_4 \cdot 7H_2O$ 0.002 g/L

P2– Dextrose (80 g/L), yeast extract (1 g/L), $NH_4C_2H_3O_2$ (2.2 g/L), K_2HPO_4 (0.50 g/L), KH_2PO_4 (0.50 g/L), $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $MnSO_4 \cdot H_2O$ 0.01 g/L, $FeSO_4 \cdot 7H_2O$ 0.01 g/L, NaCl 0.01 g/L

Experimental Carbon Source – Hydrolysate, yeast extract (1 g/L), $NH_4C_2H_3O_2$ (2.2 g/L), K_2HPO_4 (0.50 g/L), KH_2PO_4 (0.50 g/L), $MgSO_4 \bullet 7H_2O$ 0.2 g/L, $MnSO_4 \bullet H_2O$ 0.01 g/L, $FeSO_4 \bullet 7H_2O$ 0.01 g/L, NaCl 0.01 g/L

The medium was placed in serum bottles (125 mL or 75 mL) or glass test tubes (20 mL), purged with N_2 for 5 min, sealed, and then autoclaved (121 °C, 15 psig) for 30 min.

Fungal treatment

The fungi were inoculated into 80 mL fungal treatment medium. Roughly two thirds of each fungus seed culture was placed into different samples and the remaining fungi was combined and put into a third sample. This medium was then added to and 14 g corn fiber. The samples were then labeled with what fungi were present in the sample and incubated at 37 °C for 6 weeks. During the incubation the samples were flushed with air for 10 min every 5 days.

Acid Pretreatment

Corn Fiber (60 g/L) was added to acid (varying concentration and type) in an Erlenmeyer flask (250 mL). Then the mixture was placed in an autoclave (121 °C, 15 psig) for 30 min.

Cellulase hydrolysis

Enzymes AccelleraseXY and Accellulase 1500 (Genecor) were added to the corn fiber in concentrations of 0.03 ml/g biomass and 0.06 ml/g biomass, respectively. The pH was adjusted to 5 then the samples were incubated at 50 °C for 72 h. Samples were taken every 24 h.

Clostridium Acetobutylicum Seed Culture

Frozen Clostridium Acetobutylicum (ATCC 55025) cells concentrated in CGM (0.25 mL) were removed from an -80 °C Freezer and thawed. The sample was then injected into CGM (pH adjusted to 6.0) along with mineral solution.

Experimental Fermentations

The experimental fermentations were inoculated with cells from a seed culture after 18 h of incubation. The sample was then incubated at 37 °C for 72 h with samples being drawn every 24 h. The pH was maintained around 5.5. Some more details are discussed in the experimental design section.

Sample analysis

Samples were analyzed by gas chromatography (GC) and high proficiency liquid chromatography (HPLC) for concentrations of solvents and sugars/inhibitors, respectively.

Data analysis

All data was analyzed with and alpha of 0.05. The most relevant statistics may be found in appendix 1. All equations with a T or a P variable stand for *T. reesei* and *P. chrysosporium*, respectively. They were coded with (-1) standing for the absence and (+1) standing for the presence of the corresponding fungi. Batch or Trial were also coded with batch (+1) being 1 and batch 2 being (-1). These values are to be filled in algebraically for any given equations.

Experimental Design

To understand the growth behavior of the Clostridium strain being used, the optical density (OD) was measured over time. The OD corresponds to the logarithm of the cell density. The cells were grown in CGM in accordance with seed culture protocol and samples were taken every 2 to 4 hours. The OD of the samples were then plotted and analyzed. This helped determine the time when the cells should be inoculated from the growth medium into the primary fermentation.

The next part of the experiment was to screen acid type and concentration. A full factorial with two levels of acid type (H_2SO_4 and HCl) and 5 levels of concentration (0.02 M, 0.04 M, 0.08 M, 0.12 M, and 0.20 M) for the acid pretreatment was tested. The hydrolysate from the trial was then fermented. The solvent production was then analyzed. The purpose of this experiment was to determine the acid concentrations and type for the fungal pretreatment experiment.

The main experiment was a full factorial with 3 effects *T. reesei* pretreatment, *P. chrysosporium* pretreatment, and acid level. The two fungi effects were tested at two levels, presence or absence, giving four types of fungal pretreatment (none, *P. chrysosporium*, *T. reesei*, both). The levels of acid (0.00 M, 0.04 M, 0.08 M, and 0.12 M) were chosen from analysis of the previous acid screening experiment. The intent of going to such a high acid concentration was to allow the anticipated anti-inhibitor effect of *P. chrysosporium* to be observed. The following is the layout of the tables for this part of the research.

Sample Data Table				
	No Fungi	*P. chrysosporium*	*T. reesei*	Both Fungi
0.00 M HCl				
0.04 M HCl				
0.08 M HCl				
0.12 M HCl				
Control 1				
Control 2				

Table 1: Table layout

No Fungi corresponds to starting with standard corn fiber. *P. chrysosporium* and *T. reesei* correspond to using corn fiber treated by the respective fungi. Both corresponds to corn fiber treated with a co-culture of *P. chrysosporium* and *T. reesei*. All of these then underwent acid pretreatment at the specified acid level. Note that 0.00 M is plain distilled water. After the acid treatment, the samples underwent uniform enzymatic treatment at the lowest levels suggested for the brand. This was to emulate cost cutting. Figure 5 depicts the procedure as the biomass moves through the process.

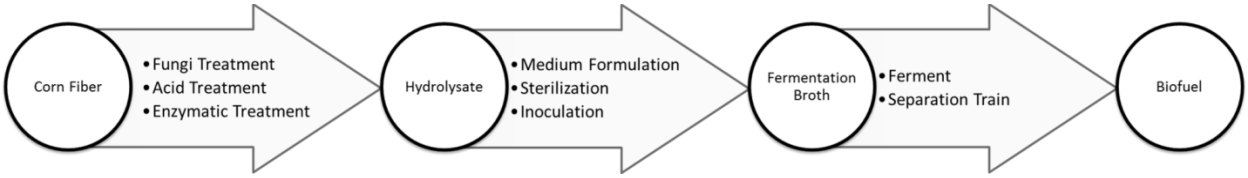

Figure 5: Biomass flow

First the corn fiber is treated with fungi. Next, the biomass was treated by acid followed by enzyme hydrolysis. Now the solution is called hydrolysate. All of this research is about the pre-hydrolysate processes. Throughout the process samples were taken and analyzed with analytical techniques.

Results and discussion

Cell Growth Kinetics

The first experiment was conducted to better understand the cell growth kinetics. It is well known that many bacterial cell cultures grow in a similar manner. This type of growth has four main phases; lag phase, growth phase, stationary phase, and death phase seen below in Figure 6.

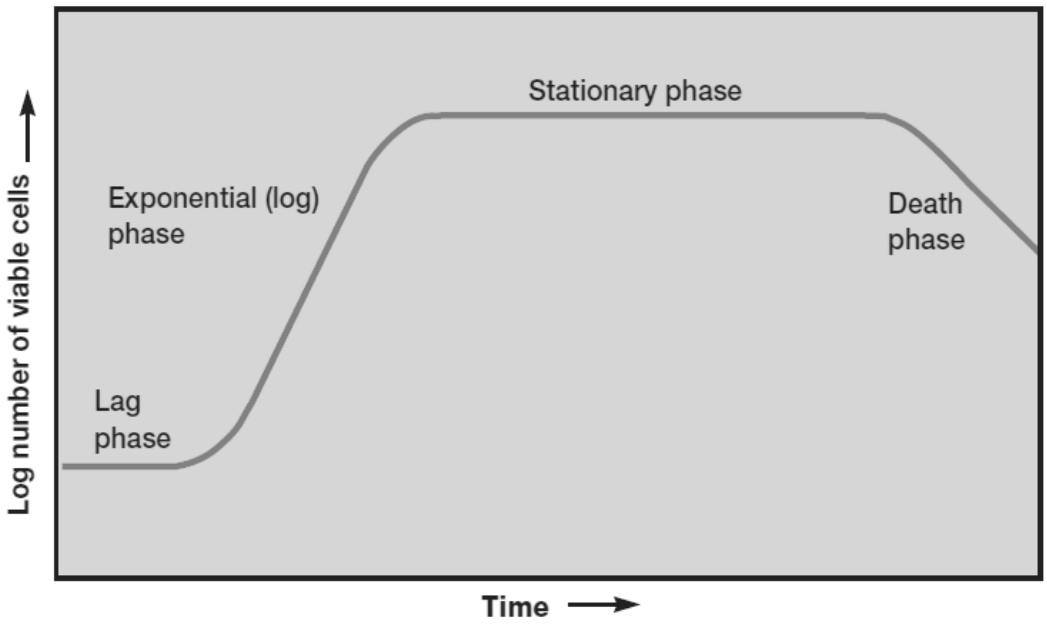

Figure 6: Microbial cell growth [12]

A similar graph was constructed using a seed culture and optical density as the measurement. It was assumed that the optical density (OD) was proportional to the log of the viable cell population. Figure 7 shows the data points along with the piece wise, best fit line.

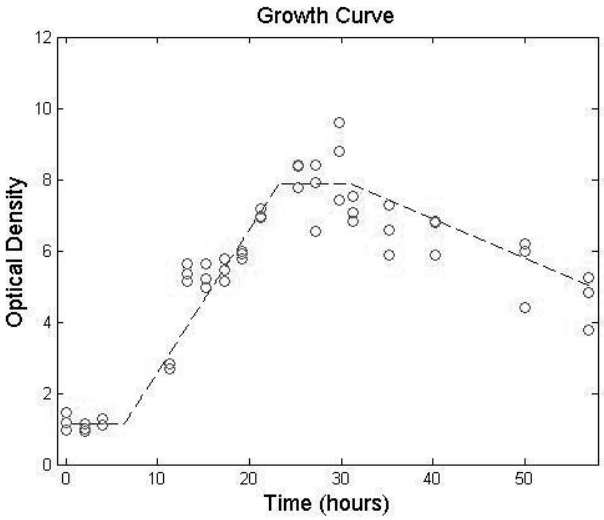

Figure 7: Seed batch cell growth

The initial line which is flat is called the lag phase. This is when the cells are not yet multiplying. The increasing line is called the growth phase. The cells are rapidly proliferating as the population increases exponentially. This stops when the population capacity is reached. The next segment is the stationary phase. This is when the cells start to produce the solvents acetone butanol, and ethanol. Next is the death phase.

It is ideal to inoculate the primary fermentation with a high cell density sample without too many products. This will give good growth and a low number of products being added to the fermentation. The data was analyzed and time ranges for the phases of the specific lineage of bacteria used in this experiment was found. The lag phase was found to last 6.38 hours. The growth phase was found to take place from 6.38 h to 23.22 h. The stationary phase then lasts from 23.22 h to 31 h. The duration of the seed culture was chosen to be 18 h to maximize the cell density while avoiding cells going into the stationary phase. Equation 1 shows the piece wise function obtained.

$$OD = \begin{cases} 1.15 & for\ \ 0.00 < time \leq 6.38 \\ 0.40 * t - 1.40 & for\ 6.38 < time \leq 23.22 \\ 7.89 & for\ 23.22 < time \leq 31.00 \\ -0.11 * t\ + 11 & for\ 31.00 < time \leq 57.00 \end{cases} \quad [1]$$

Acid Screening

In the pretreatment, acid is used. This portion of the experiment is to determine if the type of acid has any effect on the fermentation. Two types of acid, Sulfuric (H2SO4) and Hydrochloric (HCl), were used at five different concentrations (0.02 M, 0.04 M, 0.08 M, 0.12 M, and 0.20 M) in a full factorial design. Using the results to build a model, only acid concentration had a statistically relevant effect on the fermentation. Equation 2 shows the estimation for the sum of acetic acid, butanol, and butyric acid produced in g/L and equation 3 shows the equation for the amount of acetic acid produced (g/L).

$$Y = 9.7 - 32 * (Acid) \quad [2]$$

$$Y = 5.3 - \ 9.8 * (Acid) \quad [3]$$

Both show an inverse relationship with concentration of acid. This is likely to be due to the fermentation broth becoming toxic from the products of hydrolysis at higher concentrations of acid. These toxic substances are the inhibitors and were analyzed in the fungal pretreated experiment.

For the acid screening fermentation, the greatest amount of sugar (sum of glucose, *xylose*, and arabinose) used over the course of the fermentation was in the 0.02 M trials. Those were the same data points which had the greatest butanol yield. Also, above 0.04 M acid, no trial produced above 0.1 g/L butanol. This data can be seen below in Table 2: Acid Screening.

Total sugar used (g/L)					
	0.02 M	0.04 M	0.08 M	0.12 M	0.20 M
HCl	40.1	7.7	9.6	8.1	25.7
H2SO4	7.3	32.9	9.1	9.0	12.5

Butanol yield (g/L)					
	0.02 M	0.04 M	0.08 M	0.12 M	0.20 M
HCl	0.33	0.03	0.04	0.03	0.03
H2SO4	0.22	0.03	0.03	0.04	0.03

Table 2: Acid Screening

The data led to the decision of 0.12 M being the top value. This was in hopes of setting an extreme value, thus retaining the optimum configuration in the design space even if some of the growth inhibition was lessened by the fungal treatment. The other extreme was chosen to be treated with no acid. This was because the correlation between sugar in hydrolysate and acid was negative and to see if the fungi are able to replace the acid pretreatment. This range was divided four points equally separated. Next, the type of acid to use was decided. The results showed no statistical significance between sulfuric and hydrochloric acid. Hydrochloric acid was chosen because it was able to use more sugar in this screening experiment. The reason which both acids were not tested in the fungal pretreatment is the size of the experiment would have become too large.

Lignocellulose Content

The corn stover was treated with fungi as described previously. After the fungal pretreatment, the lignin and crystalline cellulose content of the corn stover cell walls were analyzed using the methods described by Foster [5] [6]. The results for the lignin and crystalline cellulose percentages can be seen in Table 3 and Table 4, respectively.

Lignin Percent				
	No Fungi	*P. chrysosporium*	*T. reesei*	Both Fungi
Trial 1	18.3%	10.5%	16.1%	12.9%
Trial 2	14.8%	11.2%	15.3%	11.3%

Table 3: Lignin content after fungal treatment

Cellulose Percent				
	No Fungi	*P. chrysosporium*	*T. reesei*	Both Fungi
Trial 1	60.0%	77.9%	40.7%	53.6%
Trial 2	64.0%	73.4%	44.8%	47.8%

Table 4: Crystalline cellulose content after fungal treatment

The data for the trials without fungal treatment seen are averages of two measurements. These measurements had a large variability, but the average value corresponds to values obtained by okridge national lab [13]. The data for the untreated corn stover was left out of subsequent analysis. After analyzing the lignin percent, *P. chrysosporium* was the only factor that had an effect on the composition. The effect was a statistical difference of over 4 percent between the presence and absence of *P. chrysosporium.* This indicates that the white rot fungi decomposed the lignin. The data failed to show a cross effect between the two types of fungi. The estimation for lignin percent can be calculated using equation 4.

$$Percent\ Lignin = 13.8 - 2.3 * P \quad [4]$$

For the cellulose percentage, both types of fungi had statistical significance in the amount of cellulose present. The presence of *T. reesei* lowered the cellulose content and *P. chrysosporium* increased the cellulose content. *T. reesei* secretes cellulase. This cellulase is likely what hydrolyzed the cellulose resulting in the decreased cellulose content. The reason that *P. chrysosporium* increased the cellulose content may be the fact that it degraded the lignin, as discussed earlier. This would take away

a portion of the lignocellulose weight while retaining the same mass of crystalline cellulose. It could have also allowed for the degradation of the hemicellulose by removing some lignin and allowing access to this more easily degraded polymer. The estimation of cellulose content is demonstrated by equation 5.

$$Percent\ Cellulose = 57.8 - 11.5 * T + 5.4 * P \quad [5]$$

After fungal pretreatment, the data has shown the expected results. The lignin degrading white rot fungi lowered the lignin content and the cellulase secreting fungi decreased the cellulose content. After the fungal pretreatment, the corn stover was then treated with dilute acid of varying concentrations. Next, this solution was charged with cellulase. The initial sugar concentration after the acid treatment and the final sugar concentration after the cellulase treatment were measured. The initial sugar corresponds to how much the fungi and acid can hydrolyze. The change in sugar was calculated and corresponds to how easy the treated biomass can be hydrolyzed via enzymes.

Enzymatic Hydrolysis

The initial sugar concentration was measured along with the final. In Table 5 are the data for the initial total sugar (glucose, *xylose*, and arabinose) concentration for the fermentation broth.

Total sugar (g/L) after acid pretreatment				
	No Fungi	*P. chrysosporium*	*T. reesei*	Both Fungi
0.00 M HCl	3.0	8.2	1.1	0.7
0.04 M HCl	15.4	11.6	12.1	10.1
0.08 M HCl	**25.5**	15.7	19.3	10.8
0.12 M HCl	20.2	18.1	17.7	**25.0**

Table 5: Sum of glucose, xylose, and arabinose present after acid hydrolysis

Some interesting observations can be made. The first is when *P. chrysosporium* is present, the initial sugar concentration does not reach a maximum and where it is not present a maximum is reached

at 0.08 M HCl. One possible explanation for this observation is that *P. chrysosporium* may secrete some product which neutralizes some of the acid. Because the acid is dilute, a small amount of basic solution could have this effect. This knowledge may prove useful when attempting to optimize the pretreatment. In conjunction with the observations made, statistics can provide an estimation for initial sugar concentration using the model in equation 6.

$$Initial\ Sugar\ Concentration\ (g/L) = 4.9 + 141 * (Acid\ Concentration) \quad [6]$$

The only factor on the initial total sugar concentration is acid concentration. This shows with more acid, more sugar will hydrolyze. This is interesting because the results from the acid screening show the higher acid results in fewer products; therefore, an optimization must be made.

Table 6 shows the initial glucose concentration.

Glucose (g/L) after acid pretreatment				
	No Fungi	*P. chrysosporium*	*T. reesei*	Both Fungi
0.00 M HCl	0.6	0.0	0.5	0.0
0.04 M HCl	2.7	1.2	1.7	0.8
0.08 M HCl	6.1	2.3	0.8	1.4
0.12 M HCl	4.4	2.6	1.6	2.1

Table 6: Glucose concentration after acid treatment

This data may appear counter intuitive. The presence of *T. reesei,* while shown to be able to reduce crystalline cellulose content, decreases the amount of glucose initially available. Equation 7 shows the estimation for initial glucose concentration.

$$Initial\ Glucose\ Concentration = 21 * (Acid\ Concentration) - 0.69 * T \quad [7]$$

This effect may be observed because *T. reesei* hydrolyzed the sugar and then metabolized it during the fungal treatment. This would suggest that the treatment time may need analyzed to optimize

the process. Next, the change in sugars from the enzymatic hydrolysis was analyzed. Table 7 shows the data for the change in total sugar during enzymatic hydrolysis and equation 8 shows the estimation equation

Change in total sugar (g/L) during enzyme treatment				
	No Fungi	*P. chrysosporium*	*T. reesei*	Both Fungi
0.00 M HCl	5.1	2.2	1.0	0.5
0.04 M HCl	7.4	12.0	7.4	7.1
0.08 M HCl	3.1	11.8	13.0	10.4
0.12 M HCl	6.4	8.7	10.0	7.8

Table 7: Change in glucose, xylose, and *xylose* with 72 h enzymatic treatment

$$Change\ In\ Total\ Sugar = 6.63 + 48 * (Acid\ Concentration) - 1196 * (Acid\ Concentration - 0.06)^2 \quad [8]$$

The change in total sugar is a downward facing parabolic equation. The derivative can be calculated and a maximum at 0.08 M HCl is found. This indicates the best acid concentration for maximizing the sugar hydrolyzed by subsequent enzyme treatment is 0.08 M. It is likely that a greater acid concentration already hydrolyzed many of the sugars thus giving a diminishing return on the enzyme treatment. This indicates that the acid or enzyme amount can be reduced. The maximum at 0.08 M HCl is interesting because the observed maximum initial sugar concentration was at the same level in the absence of *P. chrysosporium*.

The change in *xylose* was dependent on the interaction between the two types of fungi. The presence or absence of both decreased the *xylose* hydrolyzed by the cellulase. The data and estimation can be seen in Table 8 and equation 9, respectively.

Change in xylose (g/L) during enzyme treatment				
	No Fungi	*P. chrysosporium*	*T. reesei*	Both Fungi
0.00 M HCl	0.4	0.0	0.2	0.0
0.04 M HCl	-0.6	3.8	2.3	2.0
0.08 M HCl	-2.2	2.8	2.5	1.9
0.12 M HCl	-1.1	1.2	1.7	0.2

Table 8: Change in xylose during enzyme treatment

$$Change\ In\ Xylose\ Concentration = 2.8 - 0.88 * T * P \quad [9]$$

Fermentation Results

The fermentation broth was similar to the hydrolysate, but had some added nutrients. Using the concentration of each type of sugar and the corresponding molecular weight, the moles per liter of each sugar was found. From this, the total moles of fermentable sugar per liter was found. Each sugar is able to produce one molar equivalent of acetone, butanol, ethanol, acetic acid, or butyric acid. Using the moles of sugar available as the theoretical yield of these products, the percent yield can be found. The molar sugar concentrations can be seen in Table 9.

Total sugar concentration in fermentation broth (mol/L)				
	No Fungi	*P. chrysosporium*	*T. reesei*	Both Fungi
0.00 M HCl	4.75E-02	5.84E-02	1.29E-02	6.91E-03
0.04 M HCl	1.40E-01	1.49E-01	1.22E-01	1.07E-01
0.08 M HCl	1.76E-01	1.72E-01	2.04E-01	1.31E-01
0.12 M HCl	1.63E-01	1.67E-01	1.74E-01	2.08E-01

Table 9: Molar sugar concentration at the start of fermentation

The initial molar concentration of sugar is similar for the trials with at least 0.04 M hydrochloric acid. This is what was seen in the previous section. The ABE concentration produced from the fermentation can be seen in Table 10.

ABE after fermentation (g/L)				
	No Fungi	*P. chrysosporium*	*T. reesei*	Both Fungi
0.00 M HCl	2.0	0.8	0.0	0.0
0.04 M HCl	2.2	2.5	0.0	2.1
0.08 M HCl	0.0	0.0	0.0	0.0
0.12 M HCl	-	0.0	0.0	0.0

Table 10: Total ABE produced from the fermentation

Notice that above 0.08 M HCl, the ABE production was not significant. The greatest value was at 0.04 M HCl and treated with *P. chrysosporium*. For the five points that show some ABE produced, the percent yield was calculated. These can be seen in

Percent yield				
	No Fungi	*P. chrysosporium*	*T. reesei*	Both Fungi
0.00 M HCl	174%	66%	-	-
0.04 M HCl	82%	84%	-	107%
0.08 M HCl	-	-	-	-
0.12 M HCl	-	-	-	-

Table 11.

Percent yield				
	No Fungi	*P. chrysosporium*	*T. reesei*	Both Fungi
0.00 M HCl	174%	66%	-	-
0.04 M HCl	82%	84%	-	107%
0.08 M HCl	-	-	-	-
0.12 M HCl	-	-	-	-

Table 11: percent theoretical yield

The values in this table are the moles of total products over the moles of glucose, xylose, and galactose. The trial with no fungi or acid had well over 100 percent yield. This may be from some other carbon source. The detected sugar at that data point was low therefore a small difference could show large deviations. The yield at the data point where the most ABE was produced was 84 percent. At 0.04 M HCl, both samples with fungal treatment that produced substantial solvents had better yield.

Inhibitor Analysis

The data poses problems for statistics because many data points had undetectable levels. Seven inhibitors were tested using fluorescence readings in the HPLC column. Three had negligible detectable data and the other inhibitors were in very low concentrations and did not appear to have any correlation with the fungi.

Conclusion and Recommendations

From the initial acid screening, the acid type had no significant effect on the production of butanol of the amount of acids produced. What did have an effect was the concentration of acid. The greater level of acid resulted in less butanol produced. This was found to contradict the results found with the sugar hydrolyzed during the acid pretreatment, a positive correlation. A reason for this was found to be that after the enzymatic treatment, the sugar concentration was found to be about the same. This led to the conclusion that less enzymes or acid may be used.

After the fungal pretreatment, the lignocellulose content was measured. The *P. chrysosporium* was found to decrease the lignin percentage. *T. reesei* was found to decrease the crystalline cellulose percent. These were both signs that the fungi were able to secrete enzymes and break down the lignocellulose in-situ. This result was promising because the most expensive part of this process is the enzymatic breakdown of the carbohydrates. This in-situ technique could possible decrease these costs.

T. reesei was also found to negatively affect the initial glucose concentration, showing that the time of fungal treatment may need to be studied to further improve yield. The fermentation data showed that the design space may be cut back and only include acid levels up to about 0.06 M. The highest concentration of product was produced with the *P. chrysosporium* treatment. This indicates that biological pretreatment may improve the process.

Several conclusions lead to further research topics. The first is to study the effect time of fungal pretreatment has. The data showed evidence that *T. reesei* was metabolizing the biomass. If so, it is likely that the treatment time should be shorter. Another direction for the research is in minimizing the amount of commercial enzyme that needs to be used. By using the fungal culture to supplement part of the enzyme treatment, a large savings may occur.

The two types of fungi were able to break down the biomass in-situ as expected. The design space was found to be larger than needed and likely contained the optimal values. With the promising initial results, it looks like this method of pretreatment is worthy of further investigation.

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Appendix 1

This appendix has notes on the statistical analysis of the data. The abbreviations T, P, TP, Acid, and Acid2 will correspond to *T. reesei*, *P. chrysosporium*, the cross effect between *T. reesei* and *P. chrysosporium,* The concentration of acid, and the effect of acid concentration squared. The presence of the fungi can be replaced by a (+1) and the absence can be replaced by a (-1). For the batches, Batch 1 can be replaced by a (+1) and batch 2 can be replaced by a (-1). Substitute the corresponding integers to algebraically solve the model estimation. The units of all concentrations are in (g/L) for sugars and solvents and mol/L for acids unless otherwise stated. All numbers are rounded to two significant figures. Below is a brief description of the format of this statistical analysis section:

What: this section will give a brief description of what is being analyzed

Full Model: This Section will have the full list of effects in the initial model

Removal Order: This section will have the order in which effects were removed

Final Model: This will have the final fitted model

Notes (optional): This section will have any additional notes

Cell Growth Kinetics

Data from the OD was graphed and visually inspected. The data points were then set up into 4 groups. The data from time 0-4 h was sued to determine the lag phase. The points were not statistically different than the null hypothesis therefore the average was used as the intercept .The same was done for the data 21.25-35.25 h. The data point at 35.25 h was statistically different and therefore removed. The

average of the new set was used as the intercept. Then data from 11.25-21.25 h was used to fit one line, and 40.25-57 h was used to fit another. The data was then used to find four lines. The three intersections were found and those were used for the new time periods. The data was then reanalyzed, removing the data points at 13.25 and 35.25 h as outliers. The final piece wise equation was obtained.

$$OD = \begin{cases} 1.15 & for\ \ 0.00 < time \leq 6.38 \\ 0.40 * T - 1.40 & for\ 6.38 < time \leq 23.22 \\ 7.89 & for\ 23.22 < time \leq 31.00 \\ -0.11 * T + 11 & for\ 31.00 < time \leq 57.00 \end{cases}$$

Note that the data was not normalized because the variation initially was assumed to be due to measurement. Normalizing the data would then compound that difference.

Acid Screening

Before the main experiment, the type of acid was screened to see if the different acid had any effect on the fermentation. Also, the concentration was screened. The production on Acetic Acid, Butanol, Butyric Acid, and the sum of the three was analyzed. The effects were concentration, concentration squared, and type of acid. Acetic acid and total fermented were found to be statistically different than random noise.

What: The total amount of Acetic acid, butanol, and butyric acid produced

Full Model: Type of acid, Acid, Acid2

Removal Order: Acid2, Type of acid

Final Model: $Y = 9.7 - 32 * (Acid)$

What: The total amount of Acetic acid produced

Full Model: Type of acid, Acid, Acid2

Removal Order: Acid2, Type of acid

Final Model: $Y = 5.3 - 9.8 * (Acid)$

Lignocellulose

What: Percent cellulose in the biomass (cell wall) after fungi treatment

Full Model: T, P, TP, Trial

Removal Order: TP, Trial

Final Model: $Y = 58 + 11 * T - 5.4 * P$

Notes: Tukey-Kramer means comparison shown below

Level				Mean
P	A			75.65
No Fungi		B		62.00
TP		B	C	50.70
T			C	42.75

What: Percent lignin in the biomass (cell wall) after fungi treatment

Full Model: T, P, TP, Trial

Removal Order: Trial, TP, T

Final Model: $Y = 14 - 2.3 * P$

Notes: The untreated biomass was tested a second time and the averages of the two measurements were used. Also, trial was a blocking effect that was found to have no significance therefore it was removed and then reanalyzed.

Enzyme Hydrolysis

For the initial sugar concentration after acid treatment, Total sugar concentration (glucose, *Xylose*, arabinose) and the three individual sugars were tested for trends. Total sugar, Glucose, and *Xylose* showed significant trends.

What: Initial total sugar concentration after acid treatment

Full Model: T, P, TP, Acid, Acid2

Removal Order: TP, Acid2, P, T

Final Model: $Y = 4.9 + \ 140 * (Acid)$

What: Initial glucose concentration after acid treatment

Full Model: T, P, TP, Acid, Acid2

Removal Order: Acid2, TP, P

Final Model: $Y = 0.69 * T + \ 21 * (Acid)$

What: Initial *xylose* concentration after acid treatment

Full Model: T, P, TP, Acid, Acid2

Removal Order: TP, T, P

Final Model: $Y = 2.9 + 101 * (Acid) - 770 * (Acid - 0.06)^2$

For the change in sugar concentration (concentration after (72 h) – Concentration before (0 h)), total sugar concentration (glucose, *Xylose*, arabinose) and the three individual sugar concentrations were tested for trends. Total sugar and *Xylose* showed significant trends.

What: Change in total sugar concentration during the enzymatic treatment

Full Model: T, P, TP, Acid, Acid2

Removal Order: TP, T, P

Final Model: $Y = 6.6 + 48 * (Acid) - 1200 * (Acid - 0.06)^2$

What: Change in *xylose* concentration during the enzymatic treatment

Full Model: T, P, TP, Acid, Acid2

Removal Order: Acid, T, P, Acid2

Final Model: $Y = 2.8 - 5.6 * T * P$